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EXPERIMENTS IN GRAFTING

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THESE experiments were undertaken primarily to find out whether it is possible by artificial means to induce regeneration in a part that does not regenerate under ordinary circumstances; and secondly to find out what kind of a structure will regenerate from the proximal end of so highly specialized an organ as the limb of a salamander; for, while we know that from the distal end a new limb regenerates we have no information as to what will happen if the proximal end is exposed. Since the latter experiments gave more positive results they will be first considered. As it is necessary to keep the limb alive during the relatively long period required for its regeneration it was necessary to graft it, in a reversed position, on to the same animal.

The following method of grafting was employed. The leg was cut off, the skin loosened around the attached stump and turned back. The stump (composed of muscles, bone, nerves, etc.) was cut off higher up and the skin was then turned back. A pocket was thus formed. Into this pocket the piece cut off was implanted, after being turned around so that its proximal end was directed outwards. The skin was then drawn together by a ligature over the cut end. Since it is desirable to draw the skin completely over the exposed end without, however, pressing too much on the grafted piece, I have found it desirable to cut off a longer piece of the stump than will be required, and then to cut this in half, putting either half back into the pocket, which being incompletely filled, allows the skin to be drawn more readily over the end. The hind-limb being larger was used. The implanted piece, reversed in orientation, consists of bones, muscles, nerves, etc. The skin covering these parts is the old skin with normal orientation. Therefore, at least one element in the complex is not reversed.

In some respects this method of grafting proved unsatisfactory, because the cut end of the stump is so near to the graft that the new material from the stump and that from the graft may easily become mixed, so that the results would be complicated. The shortness of the grafted piece is another source of difficulty, since the short piece may become displaced during the healing of the end. To overcome these difficulties I devised another method, in which however only a single organ has its orientation reversed. The hind leg was cut off at the knee, and the femur removed from the stump for nearly its entire length. It is then shortened by cutting off part of one end, and pushed back into its original position, but with its orientation reversed. Owing to the slightly bent shape of the femur, and its much enlarged knee-end, no chance for a mistake in its orientation is possible. This procedure proved superior to the first, although the problem involved is somewhat different.

At first the large salamander, *Spelerpes ruber,* was used. This is a terrestrial form. Later the somewhat smaller *Diemyctylus viridescens* was used. Both species withstand the operation well and regenerate readily. Serial sections were made of the limbs at different stages of their regeneration.

REGENERATION FROM THE PROXIMAL END OF A REVERSED PIECE

An examination of the preparations of those cases in which a cross-section of the limb was implanted in a reverse direction in the pocket of skin of the same limb shows that as a rule proliferation takes place from the cut ends of the bones, both of the graft and of the stump of the limb. In the majority of cases it is impossible to decide with certainty whether the bones of the new limb originate from one or from both sources. While such a combined formation would be of interest in itself the conditions are such as to make it impossible to assert that both proliferations do actually contribute; for, even if the new material from both sources is continuous with the new material of the regenerating part, we can not be entirely certain that both really take part, however probable this may seem. In a few cases, however, where the skeletal material of the new limb had connected with the proliferating periosteum of the grafted piece there was every indication that the new part was derived from the exposed proximal end. In all cases that have formed a new limb, the periosteum of the cut end of the leg-bones has formed a bar of cartilage connecting their ends with the nearest ends (the original distal ends) of the bones of the grafted piece. Two possible errors make other cases of doubtful value. The short pieces of grafted bones are often turned obliquely, or even sidewise in the end of the limb, due to the contraction of the skin as the end heals, or to changes that take place in the surrounding tissues. In most other cases, the periosteal tissue growing down from the cut end of the leg-bones passes around to one side of the grafted piece, and, with or without receiving contributions from the latter, forms the cartilage of the new limb. In fact, in some cases this can clearly be seen to have taken place, in others, while not so evident, the possibility of such a process exists and renders the result uncertain.

In order to avoid this source of error I adopted the second method of grafting described above. A long piece of the femur was grafted in a reverse position in the thigh. The length of the piece prevented its rotation, and at the same time by increasing the distance between the distal end of the leg-bone and the proximal (now inverted) end of the graft it was possible to detect with greater precision the source of the material for the new bones. The presence of a single bone instead of two as in the former case also simplified the conditions. Despite these advantages, only a few cases were obtained in which the results seemed to show with great probability that all of the material for the new skeletal parts was derived from the exposed proximal end of the grafted piece. In such cases there was always established a cartilaginous connection between the cut distal end of the femur of the stump and the cut distal end of the grafted piece. The new limb is thus made up of a proximal piece of the femur, connecting cartilage, grafted piece of reversed femur, new tibia, fibula, tarsalia and phalanges from the proximal end of the grafted piece. Evidences of absorption are generally seen in the grafted piece. How far this might ultimately go was not determined.

TAIL IN LEG AND LEG IN TAIL

By means of the skin-pocket-method it is easily possible to graft a short piece of the tail (without skin) into a pocket of the leg, and *vice versa*, a piece of the leg into a skin pocket at the cut end of the tail. I have made a few such experiments with *Spelerpes ruber* not without hopes that from the graft in the leg a tail might develop, and a leg from the end of the tail. The results proved otherwise, for, whenever regeneration occurred, the new material for a new leg came from the cut end of the old leg-bones the proliferating cells passing to one side of the grafted piece and the latter becoming absorbed.

A similar process occurred in the case of a leg grafted in a tail; a new tail and not a leg regenerated. The experiment shows that care must be exercised in interpreting the other cases where the same kind of organ is grafted in a reversed position.

Regeneration after Removing a Bone some Distance from the Cut End

In order to see what would happen if the bones are absent at the cut surface, the hind leg of Diemyctylus was cut off above the knee, the greater part of the femur was removed from the stump, and the skin sewed over the cut end. Regeneration of a new leg was delayed, but took place. The stump of the leg seemed to contract somewhat, so that the cut end of the bone was brought nearer to the cut end of the muscles. The proliferation from the end of the bone must have grown down to the cut surface, and then, extending beyond this, given rise to the material for the skeleton of the new leg. The experiment shows the possibility of the same thing occurring when a grafted piece is pushed aside. Unless the graft block the proliferation from the bones of the stump, these may contribute material to the new limb.

Attempts to Induce Regeneration in the Leg of the Frog

In order to see if regeneration could be induced in adult frogs numerous experiments have been made during three winters. Pieces of the leg were cut off, and grafted in skin-pockets in various ways, but without results. I hoped that the breaking down of such grafted pieces might incite the regeneration process, if its absence in frogs were due simply to some retarding influence in the method of closure of the cut surface as occasionally occurs in pieces of other animals that have well developed powers of regeneration.

Pieces of the leg of tadpoles (without the skin) were also inserted into the leg of the frog without inciting regeneration, but as different species were probably used for graft and stock, successful results were less to be expected. Pieces of the muscles and other tissues of the tadpole's tail were inserted in skin-pockets of the leg of the frog but without effect. Since the tail of the tadpole has remarkable powers of regeneration, it seemed possible that the presence of such tissue might excite the leg to regenerate, but this did not prove to be the case. Possibly, here also, the difference in species spoiled the result, but this seems unlikely, since it has been shown that young tadpoles of different species may be readily united and form permanent unions.

It is well known that the skin of the frog has excellent powers of regeneration, yet when the leg is cut off it generally ceases further growth after the cut surface has healed over. It occurred to me that the pressure of the skin over the cut end might in itself prevent the further growth of the internal organs. To examine this possibility I cut off the fore-leg turned back the skin, cut off a piece of the stump, and then sewed up the end of the pocket. There was thus left a free space between the cut end of the stump and the end of the skin. Nevertheless regeneration of the limb was not hastened. In other cases agar, or the coagulated white of the hen's egg, was inserted into the pocket, on the supposition that as they were absorbed the regeneration might take place but nothing of the sort occurred. These experiments were undertaken before I became aware of the fact that the fore-leg of an adult frog may actually regenerate, but only after a long time and imperfectly, so that the experiments would be expected at most to facilitate or hasten the regenerative process which however they did not seem to do. In the winter of 1904–05 one frog that lived for several months, floating in the water, regenerated a long outgrowth from the cut end of a fore-leg. The new part had one or two rudimentary finger-like outgrowths, but although the outgrowth was long it was very imperfect as a limb. This frog had had its leg cut off twice, the second time two months after the first operation. In some preparations of Mr. Goldfarb I had seen the great thickening of the periosteum that takes place after several weeks, and this suggested, that if the leg were cut off anew at this time, regeneration might take place. I have also obtained more recently two or three other cases of regeneration of a limb in this way, and in these the new foot was flat and broad, and plate-like, but with scant evidence of toes. That the regeneration of a new leg is not dependent on this two-fold operation has been shown by Mr. Goldfarb who has also obtained cases of regeneration of the fore-leg of the frog after a single amputation. Since Mr. Goldfarb will describe these cases in detail further description may be omitted here.

The toad, being a more primitive member of the Anura, might be supposed to have greater powers of regeneration, but experiments have been less successful with it than with the frog. The thickening at the end of the bones forms a large knob, as it does also in the frog at first, but in the toad no subsequent changes appear to take place.

EXPERIMENTS WITH LIZARDS

During the summer of 1904, while enjoying the hospitality of the Marine Laboratory of the Leland Stanford University, I carried out many experiments with lizards, but since the results were purely negative they may be given in a few words. In the lizard the tail has the power to regenerate with great facility, but the legs appear to have no power of this sort. I tried grafting parts of the tail in skin-pockets in the legs, in the hope of inciting regeneration. In some cases large pieces were inserted, containing all of the tissues, in other cases pieces of single tissues, muscles, bone, periosteum, etc., were put into the pocket, but without producing the desired result. The lizards were kept alive in some cases for six to eight months without showing any signs of regenerating the leg during this time, but even the tail regeneration is not rapid. It appears, nevertheless, that the regeneration of the leg can not be induced by the presence in it of pieces of other parts of the body that have the power to regenerate.

CONCLUSIONS

Although the experiments undertaken in the hope of inciting regeneration in parts that do not do so ordinarily, or imperfectly and only after a long time, have not given any positive results as yet, still even the negative results are not without a certain theoretical interest. The experiments with the frog showed that the lack of power to regenerate, or only to regenerate imperfectly after a long time, is not due to the pressure of the skin on the cut end of the parts beneath. It is interesting to find that when after several months the internal parts begin to push out a new stump, as sometimes occurs, the skin is then also incited to regeneration, and will form a suitable covering for the parts beneath. It is plausible to suppose that this growth of the skin is due to the pressure of the new part from within. Other factors may also enter into the result, but that the pressure must be the main factor is shown by the failure of the skin to regenerate when other tissues, those of the tail for instance, are present, that have themselves the power to regenerate, but not finding suitable conditions for regeneration fail to form an embryonic knob. In its absence the skin is not incited to regenerate. The fact that the skin does not show any tendency to complete itself alone, although it has the power of regeneration, is important as showing that the possession of this power is not itself a stimulus that will lead to its development. Some other condition is necessary to call it forth, and in this case, that condition seems to be pressure from beneath.

Other tissues in the leg may also have the power to regenerate, but fail to do so unless certain conditions are realized. This state of affairs may lead us to hope that with a better knowledge of the conditions we may ultimately control them, and I trust this may be a first contribution towards that end.

That the muscles in the frog's leg have the power to regenerate can be shown by cutting from the side of the gastrocnemius a square piece of muscle tissue. In the course of a few months I have found that the muscle regains its size, which seems to be due, in part at least, to the formation of new muscle, although hypertrophy of the remaining fibers may also assist in the enlargement.

There is some indication that the delay in the formation of the new leg in the frog is due to conditions existing in the bones or muscles and, as I have pointed out,[1] it is significant to find that in the vertebrates the loss of power to regenerate a limb appears where cartilage has been changed to bone. The result is not however due directly to the ossification, since the new material is derived from the periosteum and not from differentiated tissue.

Especially interesting is the evidence showing that the introduction of material, itself capable of regeneration (as when the tail-tissue of the lizard is introduced into the leg-pocket) does not incite the leg to regenerate. If the process of regeneration is due to some enzyme, or other substance of this nature, that arises in an injured region, and whose presence incites the new growth, we might hope by introducing pieces of material capable of forming such substances to incite regeneration, but no such result followed. It would be unwise to lay too much weight on negative evidence of this kind, but the results as they stand indicate, perhaps, with some probability, that the primary cause of regeneration is not to be found in this direction.

Finally, to revert once more to the experiments that gave positive results. It has been shown that from the proximal end of a reversed femur new limb bones develop. This result calls for further analysis. It is clear that each level of the limb has the power to regenerate all of the parts lying more distal to it, and in all probability every level has potentially the power also to regenerate all the other parts of the limb proximal to that level. It is difficult to show that a distal part has the potentiality to produce more proximal parts, but the facts make this interpretation highly probable. How much of the distal end regenerates depends in part on its relation to what is left in the stump, and in part on the necessity of forming a distal structure. Between these limits the intermediate parts are laid down. The proximal cut end of a limb must have the same potentiality of forming distal structures as has a distal end and in those cases where the possibility exists of forming either an anterior or a posterior structure, as in pieces of lumbriculus, for example, some other relation must determine that from one end of a piece a head always develops, and from the other end a tail. I have suggested that the direction of the gradations of the old material (as expressed in their differentiation) is the factor that regulates this result. If we apply these same ideas to the special case under consideration we might expect the proximal end of the leg (or any part of it) to regenerate only proximal structures; in other words to complete the proximal end of the femur and produce a scapula at the exposed end of the leg, and, theoretically, one might imagine the further development of a salamander around the scapula as a center. The facts are the reverse. The conditions that determine in the case of the reversed femur what shall regenerate of the various possible ones are not so simple as just described. In the first place, the detachment of the femur from the rest of the limb may soon lead to changes in it that cause it to lose that gradation of materials on which the polarity of the new part depends. There is also the possibility that the polarity of the other tissues may have a counterbalancing influence. But far outweighing these possibilities there is another consideration of greater weight. The special group of tissues found in such an organ as a limb may be capable of forming only one structure, if they form anything at all, namely, a leg, and not a salamander to take the extreme case. But why always the distal end of the leg and not the proximal, *i. e.*, not femur and scapula? The determination of the distal end rather than the proximal must be due, I think, to the presence of the free rounded knob covered by the new skin which gives the stimulus for a distal structure, and the foot end of the leg is the only possible distal structure that exists for this organ.

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[1] The Harvey Lectures for 1906–7.

The case is parallel to the formation of a heteromorphic tail in the earthworm, that develops, as I have shown, from the anterior end of a piece when cut behind the level of the twentieth segment, or thereabouts. Here also a distal structure develops, but the nature of the material is such that a tail rather than a head regenerates. While polarity, as an expression of the gradation of the materials, is one of the factors that determines a result, it is not the exclusive factor. In Lumbriculus a head forms at the anterior end of a piece at nearly all levels and a tail at the posterior end. Here we must assume that the kinds of materials are so equally balanced throughout the greater length of the worm that the polarity determines the result, while in the earthworm and in the leg of the salamander another condition determines a different result. In the latter cases the kind of material, or the organ-complex, makes it possible for only a tail or a leg to develop at either end of the piece, and the presence of a free end determines that its new structure must be the terminal part, hence a foot in the case of the salamander, and a tail in the case of the earthworm, is regenerated even from a reversed end.